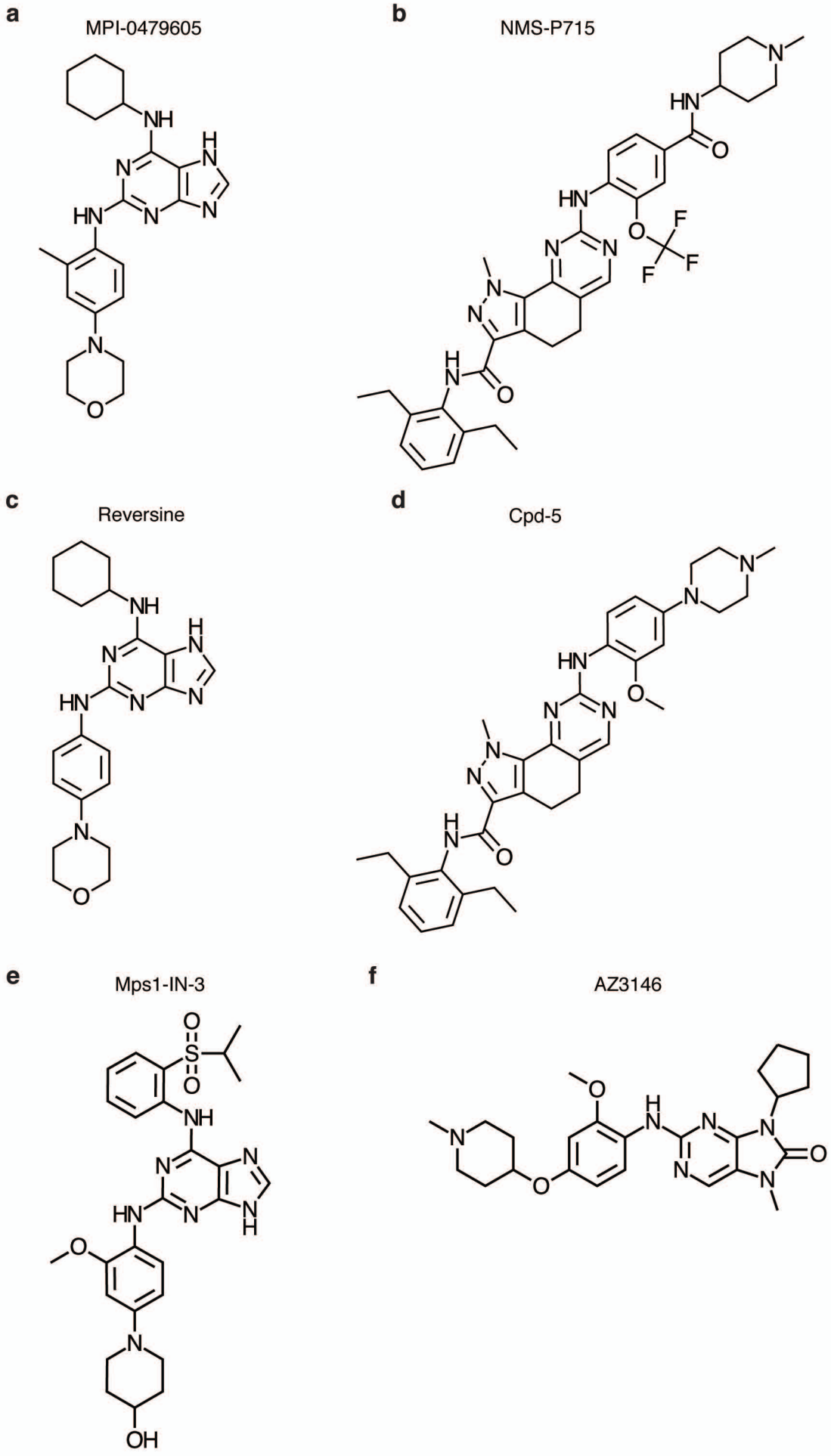
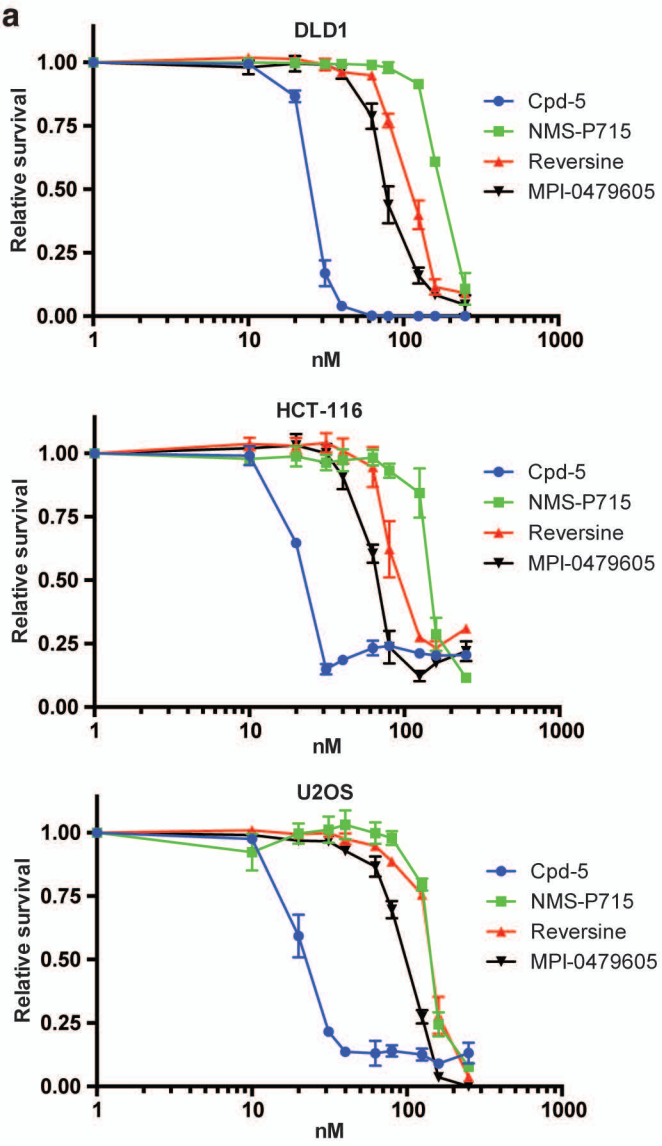
Supplementary Figure 1:



Supplementary Figure 1. Chemical structures of Mps1 small molecule inhibitors
a) MPI-0479605 b) NMS-P715 c) Reversine d) Cpd-5 (N-(2,6-diethylphenyl)-8-{[2-methoxy-4-(4-methylpiperazin-1-yl)phenyl]amino}-1-methyl-4,5 dihydro-1H-pyrazolo[4,3-h]quinazoline-3-carboxamide) e) Mps1-IN-3 f) AZ3146

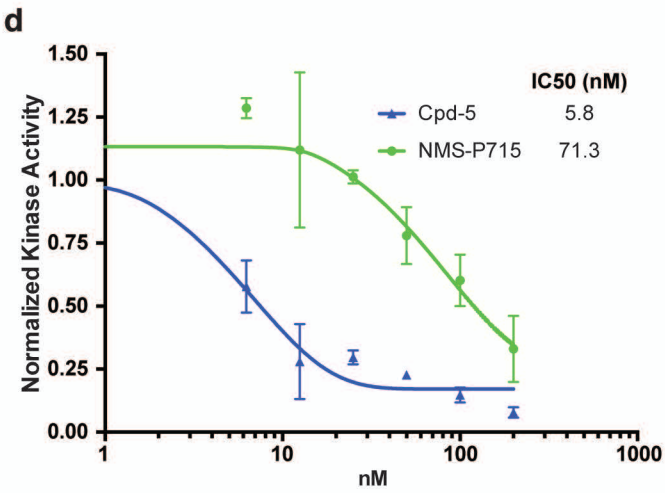
Supplementary Figure 2:

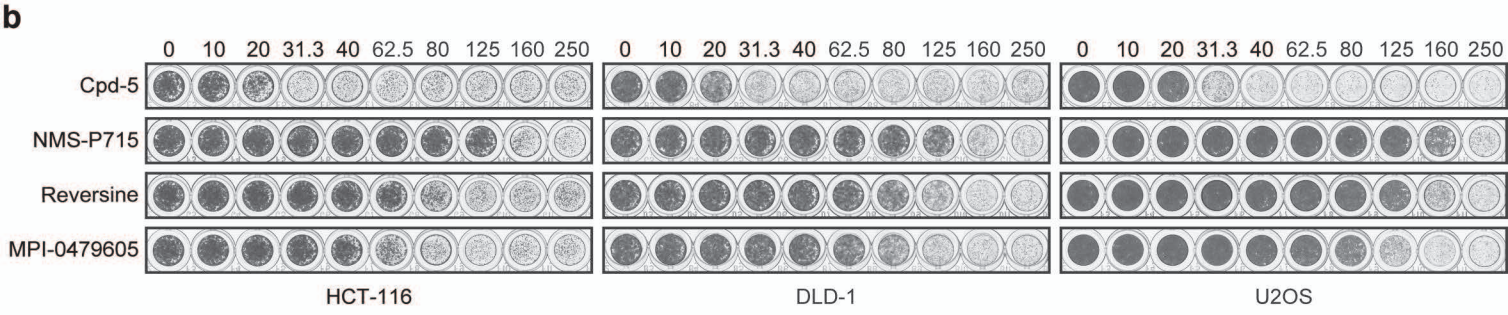


c

IC50 (nM)

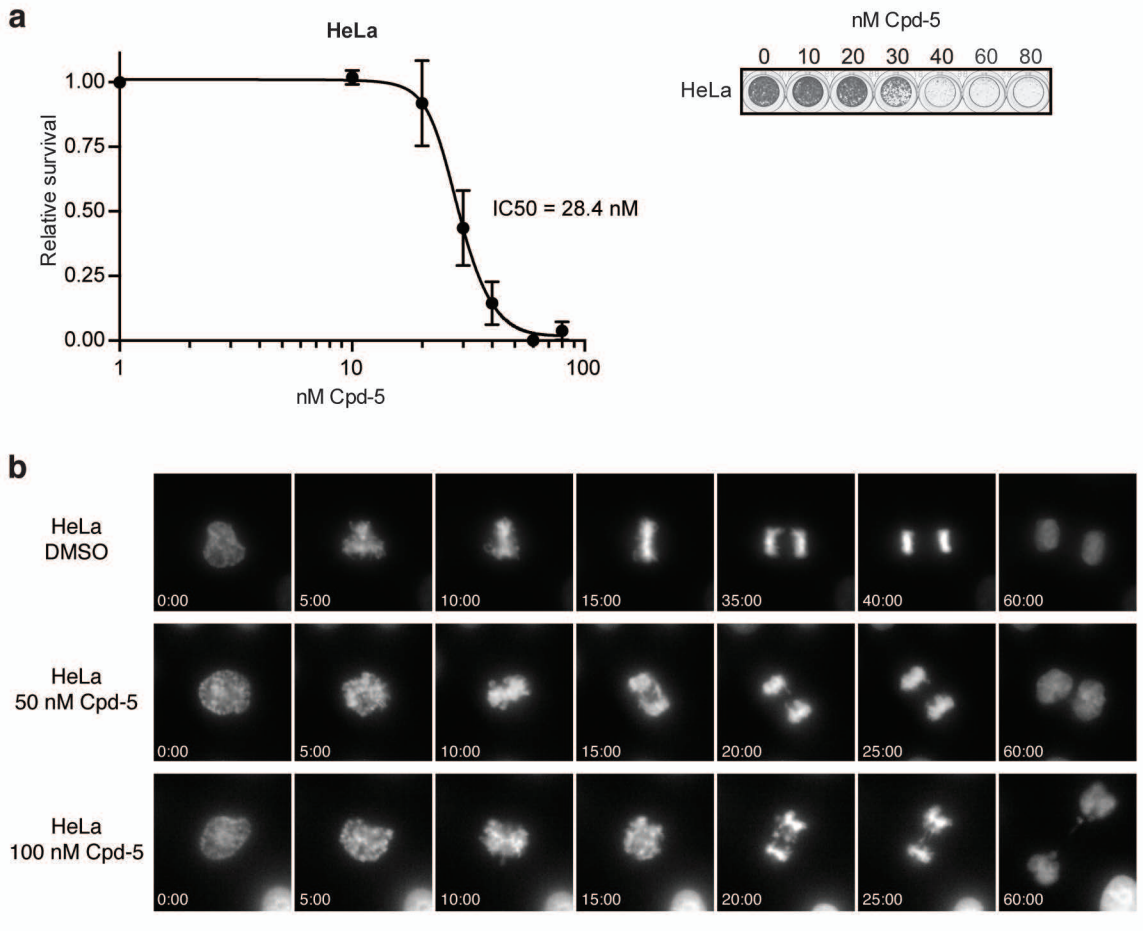
	Cpd-5	NMS-P715	Reversine	MPI-0479605
DLD1	24.6	164.8	120.3	75.4
HCT-116	20.1	153.8	78.9	63.0
U2OS	20.6	153.0	154.1	105.6





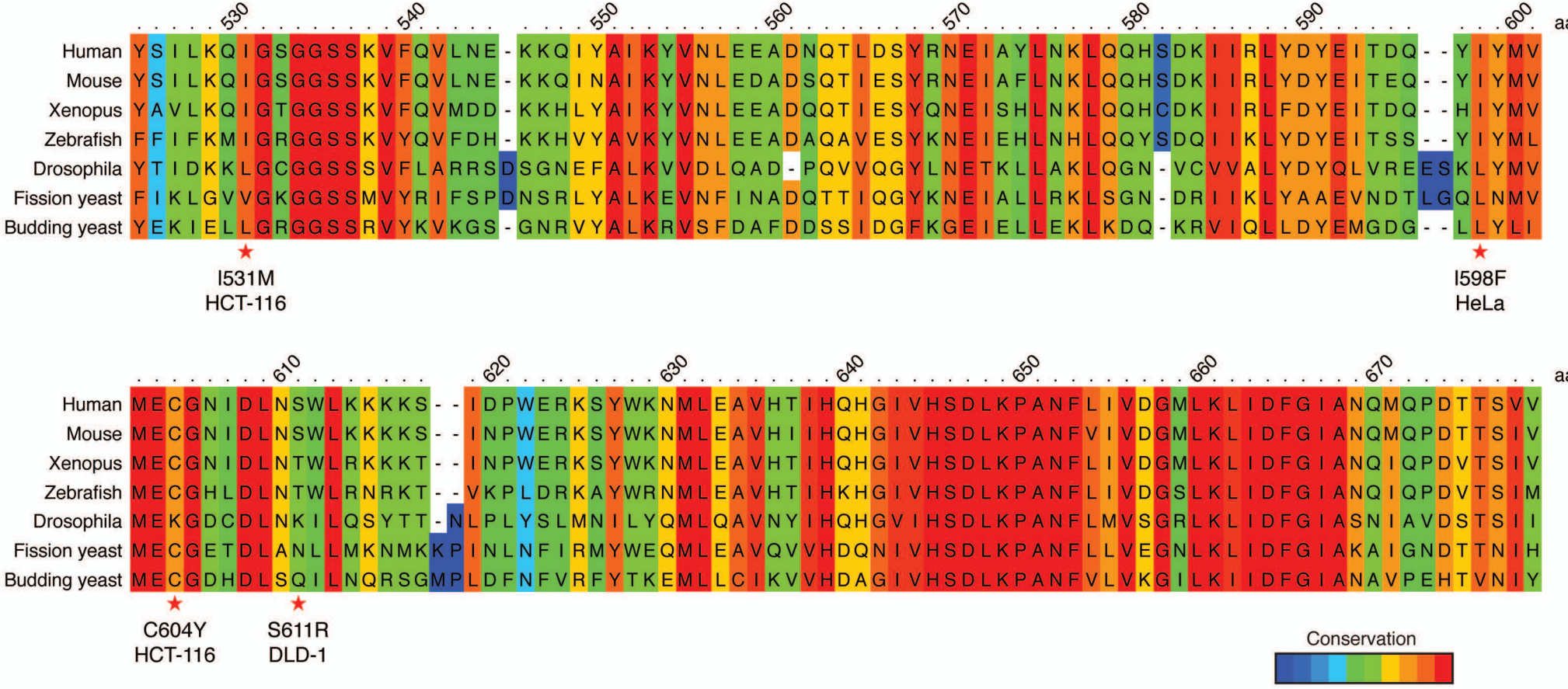
Supplementary Figure 2. Cpd-5 is a highly potent Mps1-inhibitor
a) Relative survival plots of DLD-1 (top), HCT-116 (middle), and U2OS (bottom) cells treated for 5 days with increasing concentrations of Cpd-5 (blue), NMS-P715 (green), reversine (red), and MPI-0479605 (black). Shown are the averages of three independent experiments with standard deviations. b) Representative colony formation assays of HCT-116 (left), DLD-1 (middle), and U2OS (right) cells treated for 5 days with increasing concentrations of Cpd-5, NMS-P715, reversine, and MPI-0479605. c) Table of IC50s derived from graphs in a). d) Kinase activity quantification of recombinant Mps1 upon Cpd-5 and NMS-P715 addition. Quantification was based on the signal intensities of an antibody against KNL1-phosphorylation sites. Shown are the normalized intensities of two independent experiments.

Supplementary Figure 3:



Supplementary Figure 3. Extended analysis of HeLa cells treated with Cpd-5.
a) Relative survival plots and a representative colony formation assay of HeLa cells treated for 5 days with increasing concentrations of Cpd-5. Shown is the average of three independent experiments with standard deviation and the calculated IC50 b) Representative images of HeLa-histoneH2B-YFP cells undergoing cell division after administration of DMSO or 50/100 nM Cpd-5.

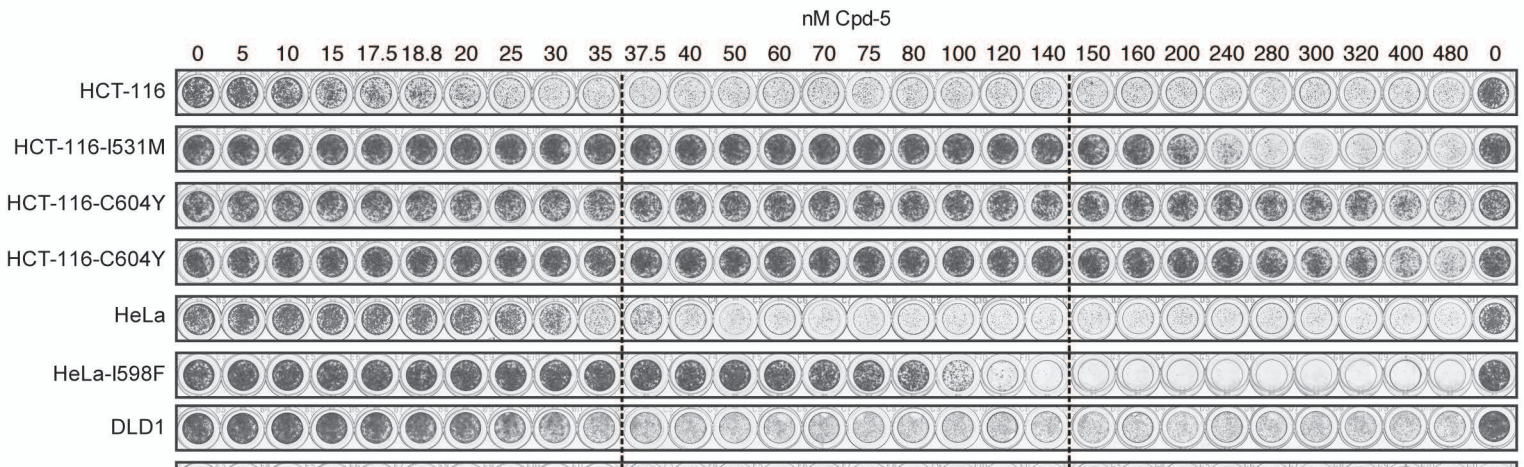
Supplementary Figure 4:



Supplementary Figure 4. Multiple sequence alignment of the Mps1 kinase domain

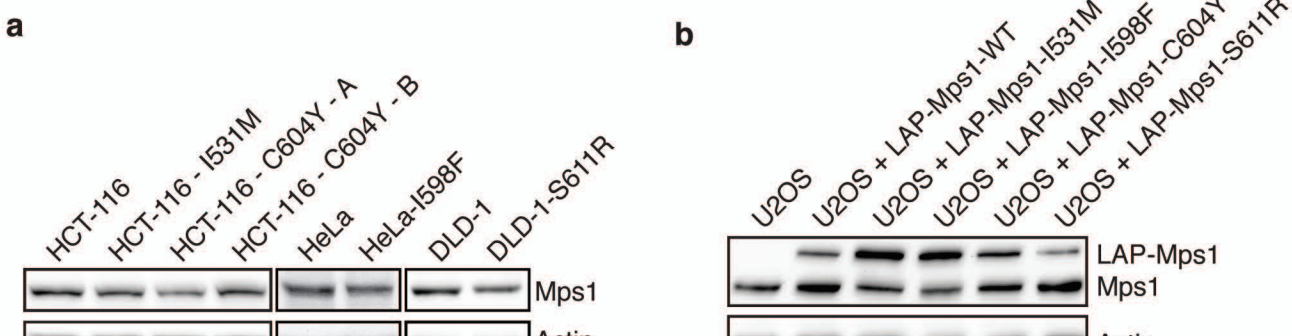
Multiple sequence alignment of the Mps1 kinase domain of representative species. A color-coded heat map shows amino acid conservation from unconserved (blue) to conserved (red). Identified mutations are marked by a star and shown below the alignment.

Supplementary Figure 5:



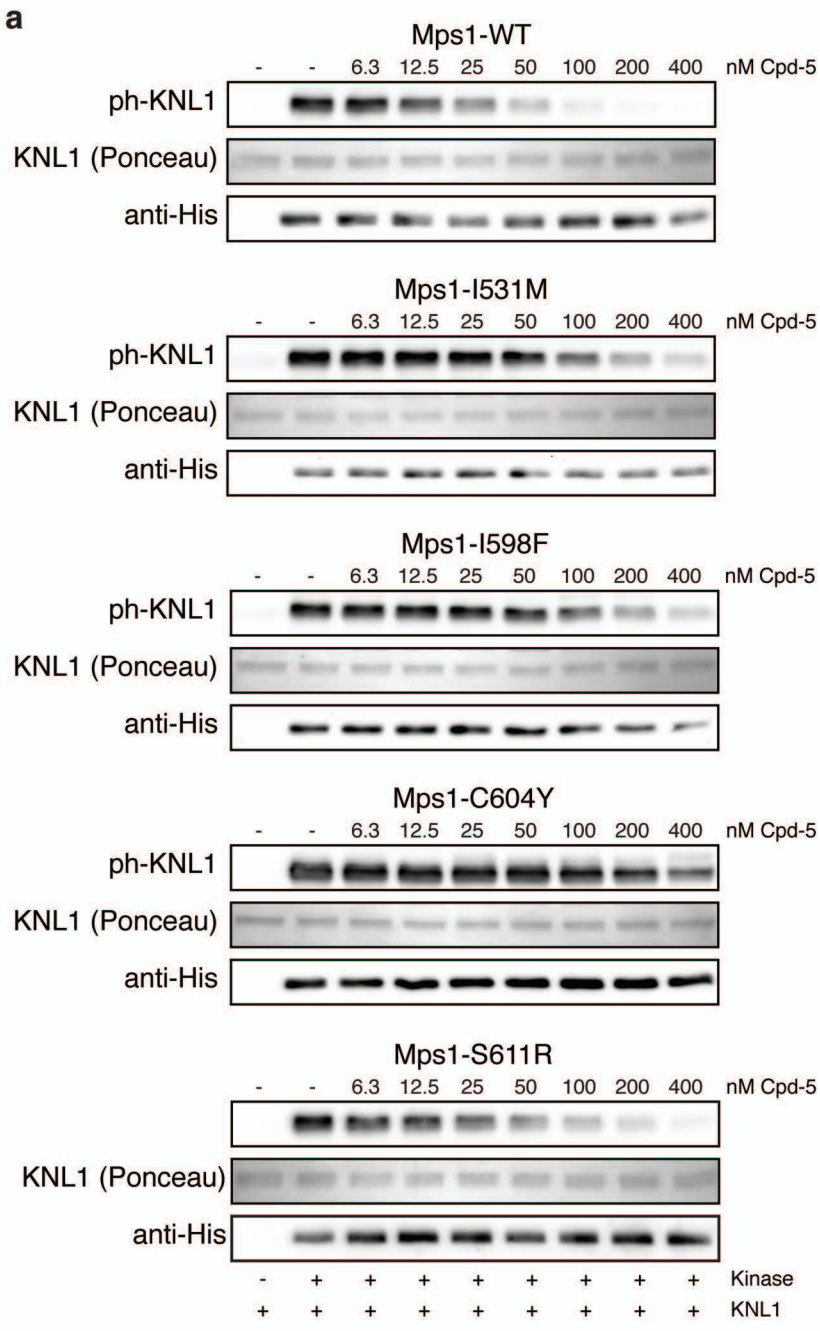
Supplementary Figure 5. Example colony formation assays for survival plots in Figure 2c
Representative colony formation assays of the indicated cell lines treated for 5 days with increasing concentrations of Cpd-5.

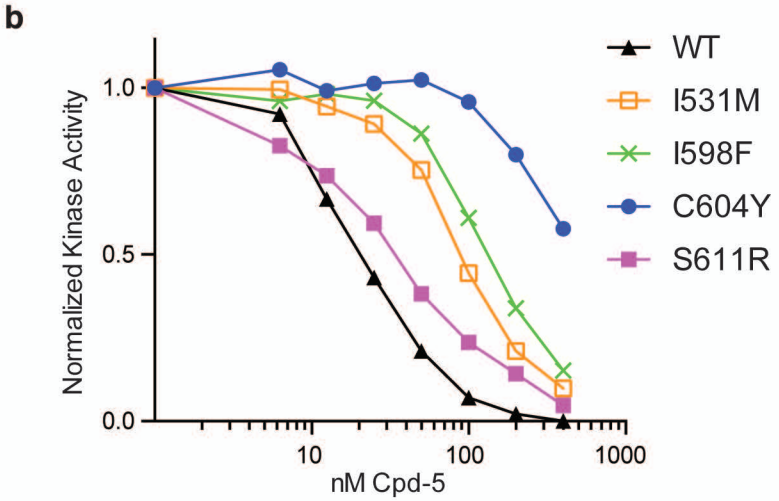
Supplementary Figure 6:



Supplementary Figure 6. Mps1 expression levels
a) Immunoblot showing the expression of endogenous Mps1 of the various wild type and mutant clones. Actin immunoblot serves as a loading control. b) Immunoblot showing the expression of endogenous Mps1 and stably ectopic expressed LAP-tagged Mps1 and mutant Mps1 in various U2OS clones. Actin immunoblot serves as a loading control.

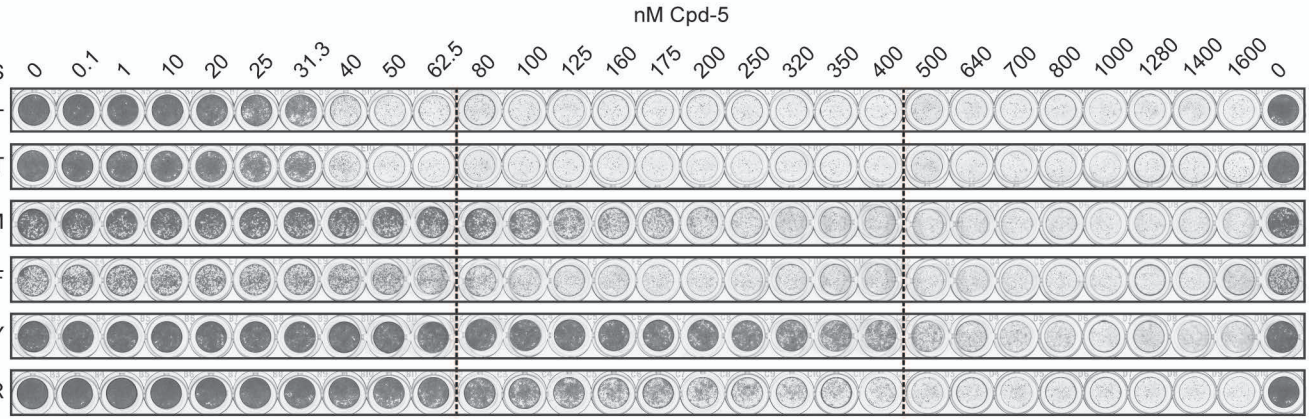
Supplementary Figure 7:





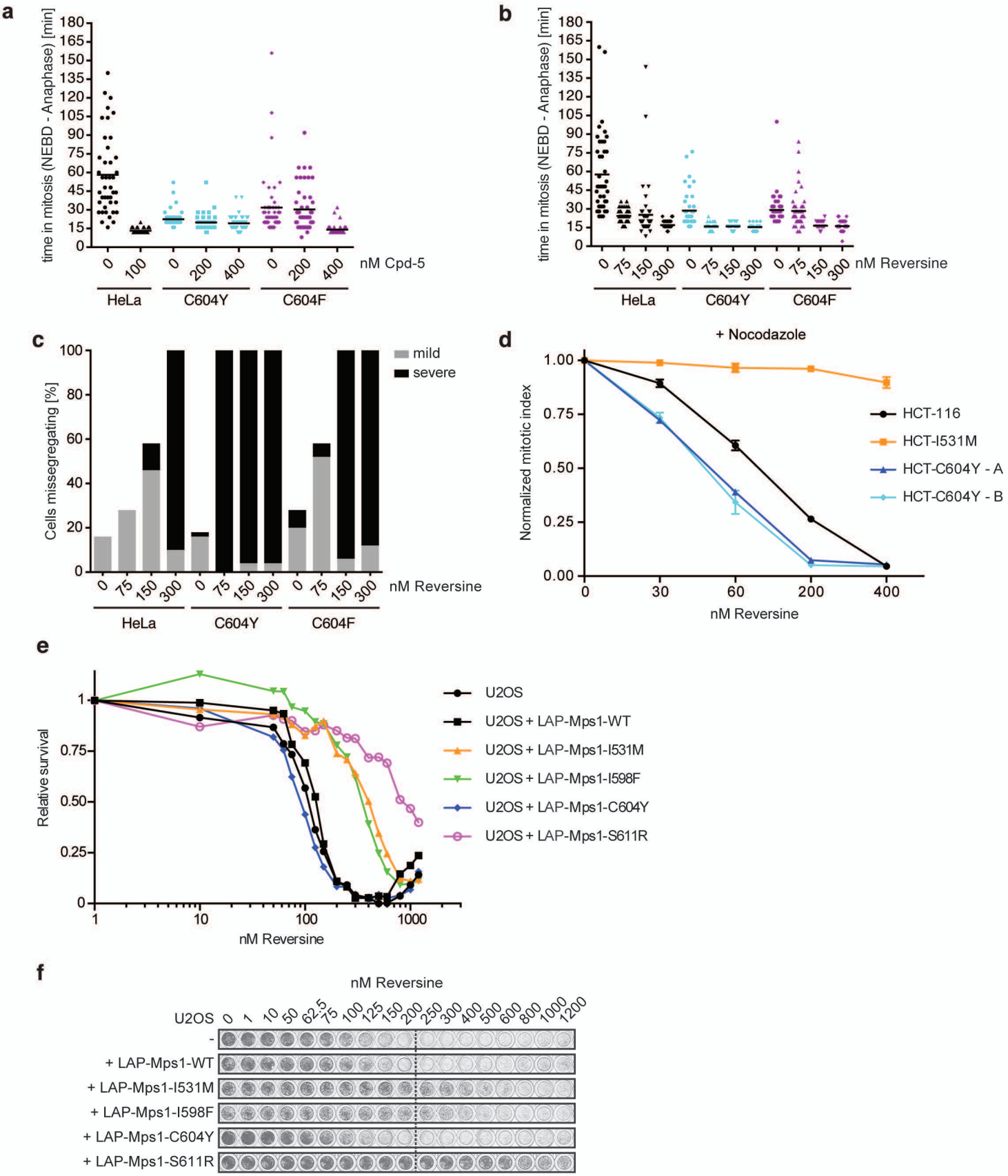
Supplementary Figure 7. In vitro kinase assays with recombinant wild type or mutated Mps1
a) In vitro kinase assays of wild type Mps1 as well as Mps1-I531M, Mps1-I598F, Mps1-C604Y, and Mps1-S611R with increasing concentrations of Cpd-5. Shown is the immunoblot of phosphorylated KNL1 (ph-KNL1) and His-tagged Mps1 (anti-His). The ponceau staining of KNL1 serves as a loading control. b) Quantification of the signal intensities of the ph-KNL1 intensities shown in a).

Supplementary Figure 8:



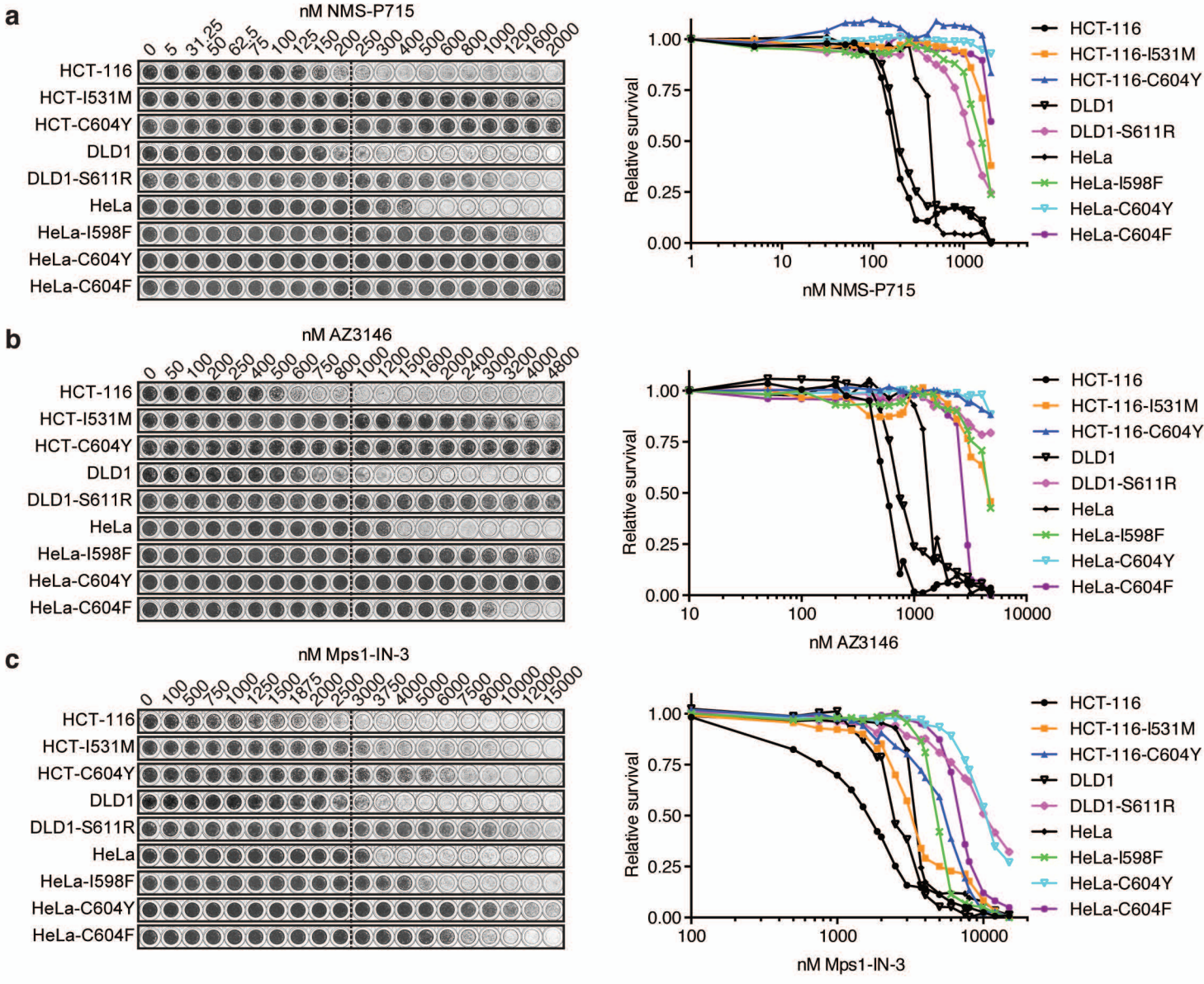
Supplementary Figure 8. Example colony formation assays for survival plots in Figure 5
Representative colony formation assay for survival plots in Figure 5. Shown are the fixed cells after staining with crystal violet. U2OS cells stably expressing LAP-tagged wild type Mps1 or mutated Mps1 were treated for 5 days with increasing concentrations of Cpd-5.

Supplementary Figure 9:



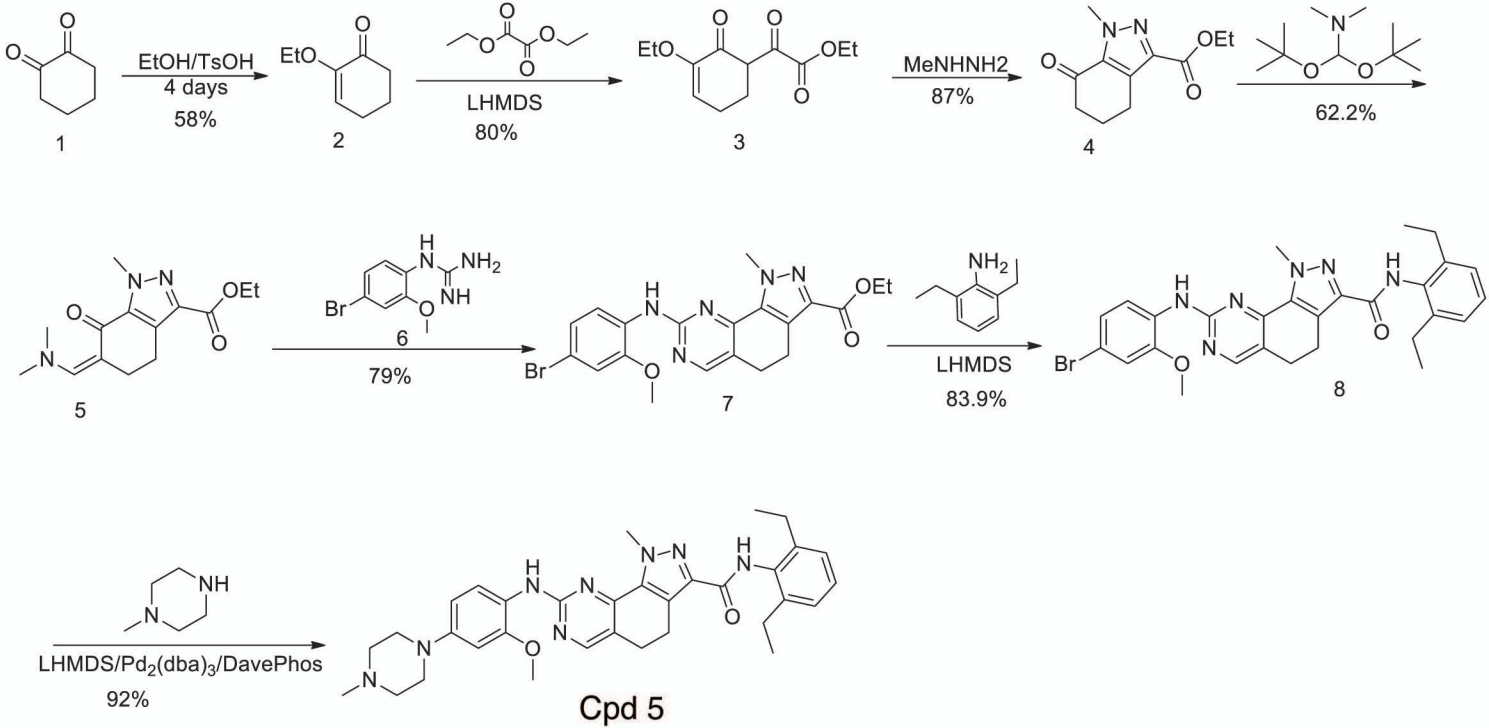
Supplementary Figure 9.
Analysis of the time from nuclear envelope breakdown (NEBD) to anaphase initiation in cells treated with various concentrations of Cpd-5 (a) or reversine (b). For each condition 50 cells were analyzed c) Quantification of chromosome missegregations after treatment with various concentrations of reversine. Two phenotypes are depicted; anaphases with mild missegregations (up to 3 chromosomes) and severe missegregations (more than 3 chromosomes). 50 cells per condition were analyzed. d) Wild type HCT-116 and HCT-116 with mutated Mps1 were treated with nocodazole and various reversine concentrations for 16 h, fixed and immunostained for phospho-H3S10. Mitotic indexes were calculated as the fraction of phospho-H3S10 positive cells over total cells (DAPI). Shown are the normalized mitotic indexes and standard deviations for various concentrations of three independent experiments.
e) Relative survival plots of U2OS cells stably expressing LAP-tag fusions of either Mps1 or mutated Mps1 treated for 5 days with increasing concentrations of reversine. Shown is the average of three independent experiments and the calculated IC50. For better visibility the standard deviations were removed from the graph. f) Representative colony formation assay for survival plots in e). Shown are the fixed cells after staining with crystal violet.

Supplementary Figure 10:



Supplementary Figure 10. Cross-resistance of the Mps1-mutated cell lines to the Mps1-inhibitors NMS-P715, AZ3146, and Mps1-IN-3.
a) (left) Representative colony formation assays of indicated cell lines treated for 5 days with increasing concentrations of NMS-P715. (right) Relative survival plots showing the average of three independent experiments. b) Same as in a) shown for the Mps1-inhibitor AZ3146. c) Same as in a) shown for the Mps1-inhibitor Mps1-IN-3. For better visibility the standard deviations were removed from the graphs.

Supplementary Figure 11:



Supplementary Figure 11. Synthesis steps for the production of Compound 5